Altered Speeds and Trajectories of Neurons Migrating in the Ventricular and Subventricular Zones of the Reeler Neocortex

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The Reelin signaling pathway is essential for proper cortical development, but it is unclear to whether Reelin function is primarily important for cortical layering or neuron migration. It has been proposed that Reelin is perhaps required only for somal translocation but not glial-dependent locomotion. This implies that the location of neurons responding to Reelin is restricted to the outer regions of the cortical plate (CP). To determine whether Reelin is required for migration outside of the CP, we used time-lapse imaging to track the behavior of cells undergoing locomotion in the germinal zones. We focused on the migratory activity in the ventricular/subventricular zones where the first transition of bipolar to multipolar migration occurs and where functional Reelin receptors are known to be expressed. Despite Reelin loss, neurons had no difficulty in undergoing radial migration and indeed displayed greater migratory speed. Additionally, compared with the wild-type, reeler neurons displayed altered trajectories with greater deviation from a radial path. These results suggest that Reelin loss has early consequences for migration in the germinal zones that are portrayed as defective radial trajectories and migratory speeds. Together, these abnormalities can give rise to the increased cell dispersion observed in the reeler cortex.

Keywords: bipolar, cortex, migration, multipolar, reelin

Introduction

Reelin protein, secreted by Cajal-Retzius (CR) cells, plays a critical role in setting up the correct “inside-out” arrangement of pyramidal neurons during cortical assembly (Rice and Curran 2001; Tissir and Goffinet 2003). Homozygous mutations in the Reelin (Reln) gene result in “outside-in” positioning of neurons in the 5 cortical layers beneath the marginal zone (MZ). This layer inversion pattern is also reflected in brains from mutant for downstream members of the Reelin-signaling pathway, which include Disabled-1 (Dab1), very low-density lipoprotein receptor, and Apolipoprotein E receptor 2 (ApoER2) (D’Arcangelo et al. 1995; Howell et al. 1997; Sheldon et al. 1997; Trommsdorff et al. 1999). Together, this signaling pathway is responsible for governing the orderly development of layered structures in many parts of the central nervous system, including the cortex, cerebellum, and hippocampus.

Despite over half a century of investigations since the reeler mouse was first reported (Falconer 1951) and over a decade since the Reln gene was identified and cloned (D’Arcangelo et al. 1995), there is still controversy surrounding the mechanisms by which Reelin controls cortical neuron layering. Two consequences of Reelin deficiency have been proposed: a defect in layering signals or a defect in neuron migration. The layer signaling hypothesis suggests that a concentrated source of Reelin emanating from a sheet of CR cells in the MZ is crucial for coordinating the orderly insertion of sequentially born neurons into 5 layers (Curran and D’Arcangelo 1998; Cooper 2008). How this is effected is a mystery. Different working models have been proposed, including rearrangement of subplate neurons to accommodate incoming neurons, chemoattraction of arriving neurons to more superficial positions, and alterations in cell adhesion (Hoffarth et al. 1995; Ogawa et al. 1995; Pearlman and Sheppard 1996; Sheppard and Pearlman 1997; Hammond et al. 2001; Nichols and Olson 2010). Reelin in the MZ may also impart a layering signal to incoming neurons, causing them to detach from radial glia and stop at the top of the cortical plate (CP) (detach and stop model, [Dulabon et al. 2000]). Recently, this model has been modified to propose that Reelin’s function is focused on inducing somal translocation of cell bodies to the top of the CP after the cell processes are anchored to the pia (detach and go model) (Hatanaka et al. 2004; Ohshima et al. 2007; Cooper 2008). Therefore, Reelin plays a critical role in effecting glial-independent somal translocation of neurons.

The neuron migration hypothesis suggests that Reelin provides a permissive cue for incoming neurons to move past preexisting neurons (Caviness 1982). Since Reelin is also expressed outside the MZ, for instance in layer V and in the intermediate zone (IZ) and subventricular zone (SVZ) (Alcantara et al. 1998; Yoshida et al. 2006), the neuron migration hypothesis does not require Reelin effects to emanate from a line source in the MZ. Indeed, ectopic Reelin expressed in the ventricular zone (VZ) of nestin-reelin transgenic mice seems capable of rescuing the preplate splitting defect in reeler (Magdaleno et al. 2002). In addition, a recent study has highlighted that functional Reelin receptors during mid- and late-corticogenesis are concentrated in cell bodies and process of the SVZ, suggesting that Reelin may exert its function while neuroblasts are still in the germinal zones (Uchida et al. 2009). So while Reelin appears to be most abundant in the MZ, its activity may be directed elsewhere during stages of migration beneath the CP. It is also noteworthy that a severe reduction of Reelin levels in the MZ is not incompatible with a properly layered cortex (Meyer et al. 2004; Yoshida et al. 2006).

Although layer inversion in reeler cortex is the more striking phenotype, it needs to be remembered there are also other abnormalities in neuron position. The reeler cortex is also characterized by blurred layer boundaries due to crossing of neurons into adjacent layers (Hevner et al. 2003; Dekimote et al. 2010). These aspects of Reelin loss have not been
properly explored, although it has been commonly assumed that these phenotypes arise pari passu with events giving rise to layer inversion. These issues assume greater importance given the recent suggestion that Reelin predominantly affects glial-independent neuron migration during the somal translocation phase (Cooper 2008). Whether or not Reelin also impacts on glial-dependent migration remains unexplored. Reelin receptors are present in the germinial zones (Uchida et al. 2009), inviting the possibility that Reelin may also govern migration of cells in the germinial zones for outcomes that are not related to layer order. This uncoupling of Reelin effects, on neuron layering versus neuron migration, has been mooted before following the observation that near total absence of Reelin has no effects on layer order, but these cortices showed more diffused neuronal positions between layers (Yoshida et al. 2006).

This study was carried out to investigate Reelin effects on cell migration in the VZ and SVZ during mid-corticogenesis. Cellular movement was monitored, and migratory characteristics quantified for bipolar and multipolar modes of migration, both of which are abundant in the germinal zones (Noctor et al. 2004; LoTurco and Bai 2006). Our results indicate that in the absence of Reelin, migrating neurons showed perturbed locomotory speeds and distorted trajectories at the onset of migration. These perturbations produce alterations in the migratory path, which may ultimately lead to increased dispersion and disorganization of neurons in the reeler cortex.

Materials and Methods

Animals

Experiments were undertaken with the approval of the Howard Florey Institute Animal Ethics Committee and conform to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Ed., 2004). Reeler (reeler) mice were bred in standard conditions, and genotyping was carried out using polymerase chain reaction (PCR) primers previously described (D’Arcangelo et al. 1996). DNA was extracted using RedExtract-N-Amp Tissue PCR Kit (Sigma), the reaction contained 0.5 mM forward and reverse primer and annealing temperature of 53 °C.

In Utero Viral-GFP Labeled Neurons

Replication-incompetent enhanced green fluorescent protein (GFP)-expressing retrovirus was produced from a stably transfected packaging cell line 293gp NIT-GFP (kind gift from F. Gage). Cells were transiently transfected with pSV-G and virus harvested as previously described (Palmer et al. 1999). Morning of vaginal plug detection was defined as embryonic day 0.5 (E0.5), and E14.5 pregnant dams were anaesthetized with Pentobarbitone and prepared for surgery. Cells were labeled in utero by injections of retrovirus-GFP into the lateral ventricle using a glass micropipette. All embryos were injected except those directly adjacent to the cervix, a technique previously shown to increase survival and reduce complications (Valacin and Tan 2003). Embryos were allowed to develop for 20–24 h after surgery before collection of brain slice cultures.

Time-lapse Imaging of Coronal Brain Slices

Embryos were collected in artificial cerebral spinal fluid (ACSF) for organotypic slice culture as previously described (Noctor et al. 2001). Briefly, dissected brains were embedded in 3% low melting point (LMP) agarose, and 400 µm coronal vibratome sections were cultured on millicell inserts in medium. Time-lapse imaging of GFP-positive cells in the somatosensory cortical region was performed at 37 °C and only one slice per brain was used. Images were captured using a 10× objective at 15-min intervals over 12 h periods.

Tracking of Neuron Migration

Each neuron was manually tracked using in-house software developed in MATLAB. If necessary prior to tracking, images were realigned in Adobe Photoshop to remove experimental drift. The position of the nucleus center-of-mass at each frame was recorded, and neurons classified with the following migratory traits bipolar morphology (associated with a radial glial fiber and having an oval shaped nucleus), multipolar morphology (no association with a radial glial fiber, multiple branches, and a round or irregular shaped nucleus), or transition morphology (changing between the 2 modes of migration). The ventricular surface was marked as a reference plane for trajectory analysis and calculation of the best-fit linear path. The tracking of somal coordinates through the imaging sequence was used to derive a number of parameters: 1) the net speed of the neuron from its start and end position, 2) the accumulated speed or overall speed of the neuron regardless of direction of migration, 3) the trajectory for each neuron in reference to the ventricular surface, and 4) deviations from a best-fit linear path.

Electroporation of Slice Cultures and Phalloidin Staining

Brains from E15.5 embryos were dissected, embedded in 3% LMP agarose, and 400 µm coronal slices sectioned and collected into ACSF. Slices were transferred to an electroporation chamber where the Chicken β-Actin promoter-green fluorescent protein (CAG-GFP) plasmid (Matsuda and Cepko 2004) was placed in the ventricle using a glass micropipette. DNA was visualized using 0.01% Fast Green. The DNA was electroporated into the brain slice with five 50 ms square pulses at 50 V administered at 950 ms intervals using the CUY21EDIT square-wave pulse generator. Slices were transferred onto millicell inserts and placed in culture media. Cultured brain slices were fixed in 4% paraformaldehyde, prepared for cryosectioning, and stained for filamentous actin using 0.5 mg/mL Phalloidin-TRITC (Sigma).

Data and Statistical Analysis

A best-fit linear path for each tracked neuron exhibiting a positive or negative trajectory from the reference plane was estimated using MATLAB. Deviations in the migratory route from this best-fit linear trajectory, denoted x and y, were determined for tracked neuron at each time frame. The deviation distance can be expressed as an average Pythagorean sum of x and y values for each neuron. Data were collected in one focal plane, thus consideration along a 3D was not required. The following analysis was performed to determine whether the data could be described with a ‘normal’ Gaussian distribution and also to identify and exclude outlying data points that could bias the analysis. Setting such a criterion provides a stricter standard to test our hypothesis. The amount of deviation expressed as the Pythagorean distance ( x 2 + y 2 ) was plotted as a frequency distribution and fitted to a standard Gaussian distribution (Battista and Kalloniatis 2002). The mean ± 3 standard deviations defined the allowable range of movement data subsequently used for analysis. A total of 9/133 data points (6%, reeler) and 5/90 data points (5.6%, wild-type) were excluded when this criterion was applied. Upon further analysis, an outlier wild-type slice was excluded resulting in the following cell numbers: bipolar wild-type n = 40, reeler n = 61; multipolar wild-type n = 37, reeler n = 63. We confirmed that the Gaussian fit was a good descriptor of the data, by undertaking a regression analysis to determine the goodness of fit value (R 2). In all cases, the R 2 value was at least 0.90, and data sets were subsequently compared using the parametric unpaired, 2-tailed Student’s t-test.

For the bin data collected from electroporation experiments, a mixed analysis of variance (ANOVA) consisting of one between genotypes and one within each genotype (cortical depth) was performed. To compare individual means, an independent samples t-test employing a Bonferroni correction with an initial α value of 0.05 was used. All other statistical analyses were performed using unpaired, 2-tailed Student’s t-test.

Results

Bipolar and Multipolar Migration in Wild-Type and Reeler Cortices

To determine whether Reelin alters the migratory kinetics of cell migration, we monitored brain slice cultures from...
wild-type and reeler embryos. Previous studies have shown that at embryonic (E) day 13, reeler embryos cannot be distinguished from their wild-type counterparts (Caviness and Sidman 1973; Caviness 1982); therefore, to investigate potential differences in the initial migration of wild-type versus reeler neurons, E14.5 brains were chosen. At this stage, the first cohort of pyramidal neurons have split the preplate (Polleux et al. 1998; Takahashi et al. 1999) into the subplate and MZ. Migration was monitored in the VZ/SVZ region to capture the initial migratory kinetics of cells in the absence of Reelin. This migratory zone allows us to determine whether either or both multipolar and bipolar modes of locomotory behavior are affected. Reelin is also expressed in the IZ/SVZ (Alcantara et al. 1998; Yoshida et al. 2006; Uchida et al. 2009) and may influence neuron migration within this region.

To visualize migrating cells born at E14.5, progenitors were infected by GFP-retrovirus following in utero injections into the lateral ventricle and embryos left to develop in vivo for 24 h before harvesting. The viral titer was adjusted to allow discrimination between individually labeled cells (Noctor et al. 2001, 2002). Infected cells in the dorsal part of the cortical wall (Fig. 1A) were identified using confocal microscopy with time-lapse capability at 15-min intervals. Infected radial glial fibers may be seen as elongated processes attached at the ventricular surface with nuclei migrating between pial and ventricular surfaces (Fig. 1B). Following division at the apical surface of the VZ, a postmitotic cell emerges with a bipolar morphology (Fig. 1B; asterisk and Fig. IC) and migrates radially along the glial fiber (Supplementary Movie 1). Previous reports have shown (Noctor et al. 2001, 2004; Tabata and Nakajima 2003; LoTurco and Bai 2006) 2 distinct forms of migrating neurons that are discernible by morphology: bipolar, where a cell has 1 or 2 processes (also known as leading and tailing processes) and attached to the radial fiber (Fig. 1C) and multipolar, where a cell has multiple processes exhibiting multiple orientations (Fig. 1B, arrow and Fig. 1D). Multipolar neurons are known to be transitory cells obtained by the conversion of bipolar cells during migration through the SVZ and IZ (Tabata and Nakajima 2003), while neurons adopt bipolar morphologies during saltatory locomotion (Nadarajah et al. 2001).

To track migration, slice cultures were monitored for up to 12 h. The position of a migrating cell in each frame was recorded with reference to the preexisting somal position, and neuron migration was recorded in the region of the VZ, SVZ, and IZ. To qualify for the study, each cell was tracked minimally for 5 h (20 frames of 15-min intervals). Despite the fact that some multipolar soma remain stationary between imaging intervals (Noctor et al. 2008), for the purpose of this study, they were classified as migratory if exhibiting extension and retraction of cell processes. GFP-positive cells whose processes were not visible in the focal plane were excluded from analysis; in addition, cells undergoing division at the VZ and SVZ at the time of tracking were also excluded. Immunohistochemical staining performed postculture revealed that a proportion of the GFP-positive cells express Tbr2 and are prospective basal progenitor cells (data not shown). Cellular morphology was the prominent criteria used for distinguishing neurons within the germinal zones.

Both wild-type and reeler cells were efficiently infected with retroviral-GFP, and time-lapse imaging showed that both bipolar and multipolar cells migrate radially (Fig. 2A,B; Supplementary Movies 2, 3). Apart from the bipolar and multipolar classification, a third category termed transition cells was also discernible, representing bipolar neurons that were seen to detach from glial fibers and begin to adopt multipolar morphologies. In a single exception, a multipolar neuron was seen to revert to the bipolar mode. Multipolar migration included neurons where, in certain instances, the soma was stationary but the processes were motile. By including this data, the "sojourning" behavior of migrating neurons in the SVZ was taken into account (Bayer et al. 1991; Noctor et al. 2004).

Quantification between the different types of migrating cells based on morphology and behavior showed no difference in the proportion of cells undergoing multipolar or bipolar migration in the wild-type slices (n = 8, 112 cells) and reeler slices (n = 4, 170 cells) (Table 1). The proportion of transition cells across different slices increased 4-fold comparing reeler with wild-type; however, this was not significantly different (Fig. 2C, P = 0.11 Student’s t-test). Bipolar and multipolar modes of migration have been reported to have different speeds (LoTurco and Bai 2006). To assess this, 2 analytical methods were used in this study: 1) net speed...
Values in slices, with a similar trend shown by multipolar neurons in (A) Bipolar (white bars) and multipolar (black bars) neurons in wild-type and transitional (gray). (B) In the absence of Reelin, the proportion of cells changing between these modes within the imaging period are classified as bipolar (white) or multipolar (black) modes of migration is not altered in the absence of Reelin. (C) The proportion of cells undergoing bipolar (white) or multipolar (black) modes of migration is not altered in the absence of Reelin. Through the VZ, SVZ, and IZ, the distribution to wild-type cells. (D) Migrating GFP-labeled cells in reeler show a similar pattern of bipolar migration with wild-type neurons. (E) In the presence or absence of Reelin (Fig. 2E, P < 0.05, Student’s t-test; Table 1). Thus, even though cells of both genotypes took about the same time to travel a similar distance, reeler cells appeared to undergo a greater degree of meandering thus covering more distance. This subtle increase accounts for approximately 10% of total distance traveled. Frequency distributions of binned accumulated speeds for wild-type and reeler neurons are shown in Figure 2F, G. These plots show a curve shift to the right for reeler, indicating greater accumulated speeds for both bipolar (Fig. 2F) and multipolar modes of migration (Fig. 2G). Quantitative analysis confirming a significant difference in accumulated speeds is presented in Table 1.

Altered Migration Trajectories and Deviations from a Best-Fit Linear Path in Reeler Cortex

In the period under study (after E14.5), postmitotic neurons are destined for the middle layers and rely on glial-guided locomotion while still in the VZ/SVZ. The final phase of migration involves somal translocation at the top of the CP (Takahashi et al. 1999; Hatanaka et al. 2004). A radial trajectory represents a direct route, however, it is accepted that locomotory cells can deviate from a straight route (LoTurco and Bai 2006). A migrating cell may shuttle forward, backward, or even sideways. Shuttling on the radial axis can be demonstrated by kymographs that portray migratory tracks from individual neurons (Fig. 3A, B). At each time interval (15-min), a strip of imaged GFP-labeled cells is compressed and compiled alongside each other to give a clear indication of somal movement (in radial direction over time) in the VZ and lower SVZ. The neuronal tracks of Figure 3A, B are displayed graphically in Figure 3C, D. These results clearly indicate that, compared with the wild-type, reeler cells have a lesser tendency to migrate uniformly and frequently move forward and backward, giving rise to irregular tracks.

Thus far, the results indicate that although the net distance covered by reeler cells is not significantly different to wild-type cells, there is greater accumulated distance covered in the reeler background. This may occur from migrating in multiple trajectories, including backward toward the VZ. This can be quantified by mapping individual soma with reference to the ventricular surface plane. Cells were categorized as migrating in a positive trajectory (away from the plane) or negative trajectory (toward the plane) or no trajectory (track does not transect the plane) (Fig. 4A–C). Migrating cells of both genotypes were individually tracked for a minimum of 5 h, and the proportions of bipolar and multipolar neurons following each trajectory quantified (Table 1). In reeler, the proportion of bipolar neurons undergoing a positive trajectory was significantly decreased compared with the wild-type (P < 0.05, Student’s t-test) (Fig. 4D). In contrast, there was no difference in the trajectories of multipolar modes of migration (Fig. 4E). A schematic depiction of trajectory tracks for a wild-type (n = 22) (Fig. 4F) and reeler (n = 23) brain (Fig. 4G) clearly shows dissimilar patterns of radial migration for bipolar or multipolar neurons. Although this is a representation of one slice, in all slices examined (wild-type n = 8; reeler n = 4), there was an obvious disorganization in the overall track pattern in reeler, with a loss of the parallel trajectory array normally seen with wild-type neurons.

The trajectory data presented thus far were calculated from the position of each cell at the start and end of time-lapse imaging. This does not take into account the degree of deviation from a radial path for a migrating cell. These deviation

Figure 2. Bipolar and multipolar cells migrate at a greater speed in the absence of Reelin. (A) Confocal image of a wild-type cortical slice showing viral-GFP infected cells throughout the VZ, SVZ, and IZ. (B) Migrating GFP-labeled cells in reeler display a similar distribution to wild-type cells. (C) The proportion of cells undergoing either bipolar (white) or multipolar (black) modes of migration is not altered in the absence of Reelin. Cells changing between these modes within the imaging period are classified as transitional (gray). (D) Graph showing no change in the average net migratory speed of bipolar (white bars) and multipolar (black bars) neurons in wild-type and reeler slices. (E) Graph showing the significant increase in the accumulated migratory speed exhibited by both modes of migration (*P < 0.05, Student’s t-test). (F) Frequency histogram of the total number of cells migrating within 2 μm increments reveals the shift in accumulated speeds between bipolar neurons in wild-type and reeler cortical slices, with a similar trend shown by multipolar neurons in (G) B, bipolar; M, multipolar. Values in C, D, and E are means ± standard error of the mean. Scale bar: A, B 20 μm.
values provide an indication of migration meandering. Two new parameters were adopted to quantify the degree of meander in either the x- or y-axes (Fig. 5A). For each track, a relationship to the best-fit linear path was calculated at 15-min intervals using time-lapse frames. The y-axis value was measured as the degree of somal movement along the best-fit path in a positive or negative trajectory relative to the ventricular plane (Fig. 5A). For each neuron, the y value was averaged for all the frames calculated for that neuron. A similar calculation was also carried out for the x value, representing the degree of horizontal deviation by the migrating soma from the predicted path (Fig. 5A). If Reelin does not alter the migratory kinetics of neurons, we would expect an identical distribution of x-y deviations for a sample population of wild-type or reeler cells in scatter plots for bipolar (Fig. 5B) and multipolar (Fig. 5C) modes of migration; this was not the case, suggesting that the reeler genotype has an effect on the meander of neurons en route to the CP. This appeared to apply to both bipolar and multipolar modes of migration.

Statistical validation was obtained by comparing the Pythagorean sum derived from the average of x and y coordinates for each cell (see Materials and Methods). Although the average movement of the bipolar cells in the reeler cortex is greater than those in the wild-type, there is no significant difference between the 2 genotypes (bipolar wild-type 3.08 ± 0.09 μm [mean ± standard error of the mean], n = 4, 40 cells; reeler 3.31 ± 0.10 μm, n = 4, 61 cells) (Fig. 5D). In contrast, there is a significant increase in movement between genotypes for multipolar cells (multipolar wild-type 2.61 ± 0.09 μm, n = 4, 37 cells; reeler 3.13 ± 0.11 μm, n = 4, 63 cells; P < 0.05, Student’s t-test). A frequency distribution of binned Pythagorean sum values portrays a greater proportion of reeler neurons with larger deviations (increased Pythagorean sum) compared with wild-type neurons (Fig. 5E).

The abnormal radial glial fibers in the absence of Reelin (Pinto-Lord et al. 1982; Hunter-Schaedle 1997; Hartfuss et al. 2003) could account for the altered trajectories observed in our study. To determine to what extent fiber orientation is altered in the cortex during the culture period, wild-type and reeler E14.5 brain slices were electroporated with CAG-GFP and cultured for 16 h (Fig. 6A,B). Electroporated neurons in both genotypes displayed strong GFP expression in the VZ and radial fibers emanating toward the pial surface. Cyrosections of cultured slices shown under higher magnification revealed radial projections from the VZ in the wild-type (Fig. 6C) and reeler (Fig. 6F) cortex. Under low magnification, the glial fiber arrangement can be observed in the wild-type and reeler cortex (Fig. 6A,B). The cortical architecture was visualized using Phalloidin-TRITC, and at higher magnification (Fig. 6D,E,G,H), the cellular alignment in the VZ/SVZ region of both genotypes did not deviate appreciably. The abnormal internal plexiform zone and obliquely oriented axonal bundles are present in the...
The analysis of cell migration in the VZ and SVZ suggests that without Reelin, both bipolar and multipolar modes of migration are present with increased meandering and speed. To test whether these observations are also reflected by neurons migrating further into cortex, CAG-GFP plasmids were electroporated into E15.5 wild-type and reeler slices. The position of GFP-labeled neurons after 40 h was binned at 75 μm intervals between SVZ and pial surface and the number expressed as a proportion of the total cell number per slice (Fig. 7E). This analysis revealed that reeler (n = 811, 6 slices) and wild-type (n = 338, 4 slices) neurons reach the CP at a similar rate (Fig. 7E; not significant, ANOVA). Thus, at least for neurons generated after E15.5, the capacity for neurons to migrate in the reeler cortex is comparable with the wild-type. Although reeler neurons move with a greater speed, the increased deviations diminish any gain in net distance traveled.

Discussion
The technique of real-time imaging is instructive for capturing the dynamic behavior of cell migration, and when applied to live cortical slices, provides the opportunity for interrogating the dynamic effects of Reelin on the migratory process. In comparison with previous studies, the current investigation has the following novelties. First, we performed detailed real-time imaging to capture the migratory dynamics of neurons in the VZ/SVZ region and compared their behaviors in the presence and absence Reelin. Second, migrating neurons were sorted into bipolar and multipolar categories to tease out differential responses by these 2 cell states to Reelin loss. Our principal
findings reveal that in the absence of Reelin, the ratio of neurons undergoing bipolar versus multipolar migration is similar to wild-type; however, reeler neurons in the VZ/SVZ tended to exhibit excessive local movement.

We classified different migrating populations in the germinal zones of wild-type and reeler neocortex based on their morphologies. It was not possible to distinguish which cells migrating from the VZ become basal progenitors in the SVZ. A recent study suggests that a distinction can be made between multipolar cells and basal progenitors through mode of migration and migratory speed (Tabata et al. 2009). Tabata and colleagues showed that basal progenitors exit the VZ at greater speeds with somal translocation morphology, whereas multipolar cells migrated slower and accumulated in the lower part of the SVZ prior to migration toward the CP. In our analysis, a bipolar cell exiting the VZ was classified as having 2 process extensions parallel to the radial fiber, thereby undergoing locomotion. If correct, this would imply that our bipolar cells would indeed become multipolar neurons and are not basal progenitors.

These results throw new light on Reelin function suggesting that it influences the earliest steps of neuron migration in the VZ/SVZ. A current view suggests that without Reelin signaling, neurons migrate slower compared with their wild-type siblings and, consequently, occupy deeper positions in the CP (Cooper 2008). Our earlier work with chimeras also reported the tendency of mutant Dab-1 neurons to remain in the lower part of the cortex compared with their wild-type siblings (Hammond et al. 2001). This interpretation was supported by observations in which neurons tended to stop migration along the borders of the IPZ (Tabata and Nakajima 2002); however, the above conclusions were based on end-point analysis of fixed material. Although failure of somal translocation may generate the above outcomes, it may also arise from failure of migration in the germinal zones. We have used real-time imaging, which provides an accurate assessment of migratory behaviors and showed that the absence of Reelin causes excessive meandering of locomoting neurons. In our view, failure of Reelin signaling does not necessarily incapacitate neuron migration, it might hasten migration in neurons and cause loss of radial directionality due to altered trajectories.

The above results suggest that apart from Reelin activity on postmitotic neurons in the CP, Reelin might also impart migratory cues during the earlier phases of migration in the VZ/SVZ. These arguments are supported by accumulating evidence that additional Reelin sources and Reelin receptors are remotely sited, away from the MZ. Reelin protein is expressed in the IZ/SVZ of E15.5 cortices, providing a proximal source for neuroblasts and migrating neurons in the germinal zones (Alcantara et al. 1998; Yoshida et al. 2006; Uchida et al. 2009). Furthermore, premigratory cells in the VZ express mRNA for the Reelin receptor ApoER2 and adapter protein Dab-1 (Howell et al. 1997; Rice et al. 1998; Trommsdorff et al. 1999; Luque et al. 2003). More recently, compelling evidence for Reelin function in the germinal zones was provided by the demonstration of functional Reelin receptors in these regions (Uchida et al. 2009). Although the study by Uchida et al. (2009) identifies the presence of receptors binding to the Reelin fragment R3–6, it is possible that Reelin function in the germinal zones is exerted through modulation of other signaling mechanisms including Integrins, Notch, or the amyloid precursor protein (Dulabon et al. 2000; Hoe et al. 2006; Hashimoto-Tori et al. 2008; Marchetti et al. 2010). Together, the above point to Reelin activity on early migrating neurons in the VZ/SVZ that may be separate to mechanisms giving rise to neuronal order in the CP. Thus, disturbed migratory activity in reeler, expressed as aberrant speeds and trajectories, could be magnified as these neurons reach the CP and be detected as widespread dispersion patterns with distortion of layer boundaries.

Attention should be drawn toward a number of caveats that may temper our conclusions. First, our analysis was undertaken during mid-corticogenesis, focusing on the middle layer neurons in reeler cortices. Therefore, it is possible that our observations may be secondary to loss of preplate splitting (Caviness and Sidman 1973; Polleux et al. 1998) or the stalling of earlier born neurons and the formation of the IPZ in the reeler cortex (Pinto-Lord et al. 1982; Tabata and Nakajima 2002). However, the physical barriers of last 2 features would impose a near stoppage of incoming neurons, which we did not observe. On the contrary, we frequently record neurons locomoting at greater speeds and invariably exhibiting a meandering path en route to the CP.

Second, Reelin has been shown to be important for normal radial glia development in the cortex and hippocampus (Hunter-Schaedle 1997; Förster et al. 2002; Hartfuss et al. 2003; Luque et al. 2003; Nomura et al. 2008). In the reeler cortex, process extension of radial glial fibers is impaired and the end feet terminate before reaching the pial surface (Hunter-Schaedle 1997; Hartfuss et al. 2003). This may have implications on neuron migration, particularly in the outer cortical zones. By contrast, our analysis was conducted in the
germinal zones where cellular architecture does not deviate appreciably from the wild-type cortex (Fig. 6; Hunter-Schaedle 1997); however, it needs to be appreciated that glial scaffold disruption in the upper aspects of the CP may alter migratory trajectories in the germinal zones which we observed.

Third, removal of Cullin 5 (E3 ubiquitin ligase component involved with Dab1 degradation) from E12.5 neurons increases their speed of somal translocation due to excessive Dab1 activity (Feng et al. 2007). This is at odds with the current observation, where loss of Reelin signaling is associated with increase migratory speeds of E14.5 neurons in the VZ/SVZ. One reason for this difference is that Feng et al. (2007) studied glial-independent somal translocation in neurons approaching the MZ, whereas the current study is focused on glial-dependent locomotion in the germinal zones. It is likely that Reelin signaling has multifaceted outcomes depending on the modes of migration in different parts of the cortical depth and different stages of corticogenesis.

In conclusion, we have used real-time imaging to analyze the behavior of migrating neurons in the VZ/SVZ of reeler cortex. We demonstrate that compared with the wild-type, cells in reeler exhibit primary migratory defects in speed and trajectory from the earliest stages of their locomotion. We provide quantitative evidence that Reelin is required for locomotion of both bipolar and multipolar neurons in the radial direction. This suggests that in the absence of Reelin, these migratory

Figure 7. Overall migratory distance within the cortex is comparable between wild-type and reeler neurons. (A) Confocal image showing wild-type and reeler (C) coronal cortical slices from E15.5 embryos cultured for 16 h after electroporation with CAG-GFP. The soma of neurons can be seen migrating along the radial glial fibers from the VZ to pial surface (P). (B) After 40 h in culture, GFP-positive neurons are positioned throughout the cortical depth for wild-type and reeler slices (D). (E) Binned-distance (75 μm) histogram of the distribution of GFP-positive soma in the region between the SVZ and pial surface for wild-type (blue) and reeler (red) slice cultures. This confirms the ability for reeler neurons to migrate toward the CP (NS, ANOVA). Values in E are means ± standard error of the mean. Scale bar: A–D 50 μm. NS, nonsignificant.
defects lead to amplification of abnormal neuron positions in the adult cortex that may be separate from other deficiencies giving rise to layer inversion.

**Funding**


**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

**Notes**

We would like to thank Fred Gage for the retroviral-GFP construct and packaging line; Connie Cepko for the CAG-GFP construct, Robert Hevner for the Tbr2 antibody, and Ben Thompson for helpful discussions on the statistics. **Conflict of Interest:** None declared.

**References**


